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Long-term durability of porous hydroxyapatite with low-pressure system to support osteogenesis of mesenchymal stem cells

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Abstract. In this study, we hypothesize that loading more marrow-derived mesenchymal stem cells (MSCs) into porous material by using a low-pressure system during subculture, creating a composite which combines MSCs and a novel mechanical reinforced porous hydroxyapatite, can result in more bone tissue formation *in vivo*. Within 26 weeks postimplantation, we examined *in vivo* bone formation of the experimental group with 100 mmHg pressure applied to porous HA blocks loaded with MSCs. For *in vivo* testing, the 2-week subcultured HA/MSC composites were implanted into subcutaneous sites of syngeneic rats. These implants were harvested at 13 and 26 weeks after implantation. SEM showed that the pore surface is covered by osteoblasts as well as collagenous extracellular matrix at 13 weeks. Light microscopy revealed the quantity of bone at 26 weeks was greater than at 13 weeks. These results showed that the novel mechanical reinforced porous HA combined with MSC has more potential for bone formation at 100 mmHg, making this method very efficient for bone reconstruction.

Keywords: Hydroxyapatite, mesenchymal stem cells, low-pressure system, tissue engineering

1. Introduction

Autogenous bone graft has been considered the golden criterion for treating bone defects in orthopaedic surgery. However, this does have some problems: a second operation, risk of infection, the

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amount and quality of the bone graft. Therefore, to reduce the risks of a tissue-based graft, artificial synthetic porous hydroxyapatites have attracted attention in the field of tissue engineering as a bone graft substitute [1–10].

There are still some limits to their application, including the low mechanical properties of hydroxyapatite, poor interconnectivity of pores, and methods to induce more bone formation in porous hydroxyapatite. Moreover, in some bioceramics, resorption of newly formed bone has occurred after 13 weeks implantation [2]. Moderate compressive strength of porous ceramics is necessary in some clinical applications, good interconnection benefits vasculature invasion, and blood supply guarantees further growth of bone tissue in porous hydroxyapatite. In our previous study, we observed that using a low-pressure system during subculture, more marrow-derived mesenchymal stem cells (MSCs) can be loaded into porous material, and has more potential for bone formation at 100 mmHg [6]. In this study, we got good results of *in vivo* bone formation with a low-pressure system over long period.

2. Materials and methods

2.1. Making novel porous hydroxyapatite

The novel sintered porous hydroxyapatite (HA) was prepared as reported previously [11]. Briefly, the HA slurry was foamed by adding polyoxyethylenelaurylether (PEI) and mixing. PEI was used as a dispersion agent for HA powder. Sixty grams of HA calcined powder were dispersed in 40 g of 15 wt% polyethyleneimine aqueous solution by stirring and sonication. The slurry was foamed by adding 1 g of polyoxyethylenelaurylether and mixing. Diepoxy compound was then added to the foam as a cross linking agent. The pores were fixed by cross-linking PEI with diepoxy compounds and the HA porous body was sintered at 1200°C for 3 hours.

The HA sintered porous body had a high porosity (77%) and was completely interconnected. Average pore diameter was 500 μm and the interconnecting paths were 200 μm in diameter. The compressive (17 MPa) and three-point bending (7 MPa) strengths were high (Fig. 1). High interconnectivity was shown on this picture.

2.2. Bone marrow-derived MSCs culture

Marrow-derived MSCs were obtained from Fischer 344 male, 7-week-old rats, according to the methods of Maniatopoulos [12]. The femora were excised aseptically, cleaned of soft tissues, and washed 3 times, for 5 min each, in 6 ml of standard culture medium. Then, both epiphyses were removed, and the marrow was flushed out with 10 ml of culture medium expelled using a syringe with a 23-gauge needle. The obtained cell suspension was distributed into T-75 culture flasks (Falcon, Franklin, Lincoln Lakes, NJ) with 15 ml of standard medium and incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. The standard medium consisted of Eagle's Minimal Essential Medium (MEM) containing 15% fetal bovine serum (ICN Biomedical Japan Co. Ltd.) and antibiotics (1 × antibiotic-antimycotic, including 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B, GIBCOBRL, Life Technologies, USA). The medium was changed 24 h later to remove non-adherent cells. The remaining adherent cells left were mainly MSCs [11–14]. The medium was replaced with fresh medium every other day.

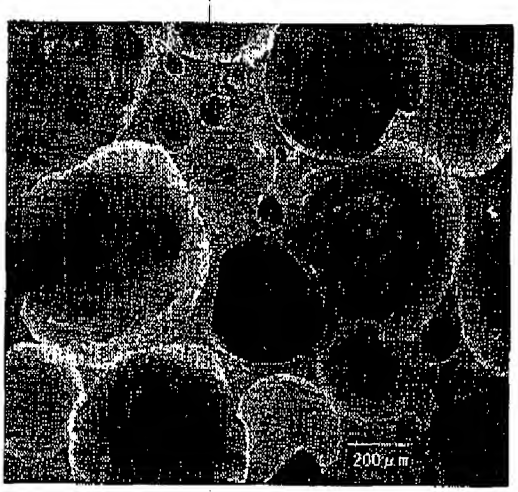


Fig. 1. SEM photomicrograph of the microstructure of the novel synthetic porous hydroxyapatite. Bar = 200 μm.

2.3. *Loading MSCs into porous HA by low pressure system*

MSCs were cultured *in vitro* for 10 days until confluent. Then, the cultured dishes were treated with trypsin and concentrated to 10^6 cells/ml. The porous HA ceramic blocks were then soaked in a cell suspension within the culture dish. The dish was put inside the vacuum desiccator at low-pressure using different low pressure values. The low-pressure system was used as reported previously [6]. Briefly, it consisted of an Ulvac G-5 oil rotary vacuum pump (Sinku Kuko, Japan) and Iuchi Polycarbonate vacuum desiccator (Tokyo Rikakikai) connected to each other by rubber and silicon tubes. We examined experimental group with 100 mmHg pressure applied to porous HA blocks loaded with MSCs.

After treatment with 100 mmHg pressure, the culture dish was incubated for 2 weeks. The medium was renewed every other day.

2.4. *Surgical procedure*

For *in vivo* testing, syngeneic 7-week-old male Fischer rats were anesthetized by intramuscular injection of pentobarbital (Nembutal 3.5 mg/100 g BW) following light ether inhalation. Six subcutaneous pouches were created in the back of the rat for HA/MSCs composites implantation. HA/MSCs composites from the 100 mmHg and normal pressure groups after 2 weeks of subculture were implanted on the back of each rat. The composites from normal pressure group were taken as control group. There were 36 HA/MSCs composites in each group.

2.5. *Light microscopy*

For light microscopic observation, the composites were fixed in 10% buffered formalin, decalcified, dehydrated in a graded series of alcohol solution, embedded in histparaffin, and stained with hematoxylin and eosin. These specimens were observed under an optical microscope.

2.6. Scanning electron microscopy

For SEM, the composites were evenly cut at the central part of the HA block. Specimens were fixed with 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.4), postfixed with 0.1% osmium tetroxide, dehydrated in a graded alcohol series, and dried in a critical point dryer. The composites were sputter-coated with platinum in an ion coater, and observed with an SEM.

3. Results

Light microscopy revealed that the earliest bone formation occurred at 2 weeks postimplantation, and mature bone formation in the HA/MSCs composite at 4 weeks postimplantation. Figure 2 shows bone formation in HA/MSCs composites 13 weeks after implantation. Original magnification ×40. B indicates osteocyte. P indicates pore. Arrow indicates new-formed marrow cells. Mature bone tissue in the porous area is observed. There were blood vessels existing in the pore areas. Bone formed in HA directly interfaced with the pore surface of HA without fibrous tissue intervening. The quantity of bone at 26 weeks was greater than at 13 weeks postimplantation in Fig. 3. Figure 3(a): Much mature bone tissue in the porous area is observed. White area shows the ghost of hydroxyapatite ceramic produced by decalcification. Original magnification ×40. Figure 3(b): Higher magnification of the rectangular area in Fig. 3(a). Plenty of blood vessels existed in the pores. P indicates pore. C indicates mature osteocyte. O indicates active osteoblast forming bone. B indicates blood vessel. Original magnification ×100.

However, not all of the composites in the normal pressure group showed satisfactory bone formation.

In the SEM study, mineralized collagenous extracellular matrix as well as active osteoblasts was observed in the HA/MSC composite at 13 weeks after implantation (Fig. 4). Figure 4(a): The pore surface is covered by osteoblasts as well as collagenous extracellular matrix. The pore surface is almost covered by rough and round cells as well as collagenous extracellular matrix. Bar = 20 μm. Figure 4(b): Higher magnification of the large rectangular area in Fig. 4(a). O shows osteoblasts which seem to be active, can be seen on the surface of HA. C shows collagenous extracellular matrix. Bar = 3 μm.

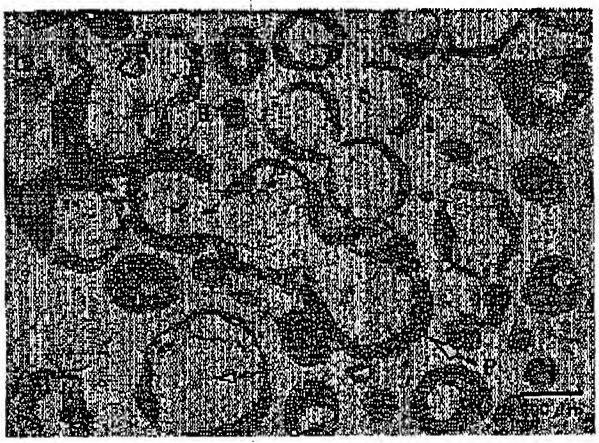


Fig. 2. MSCs-subcultured HA composite 13 weeks after implantation. Hematoxylin and eosin stain. Original magnification ×40.

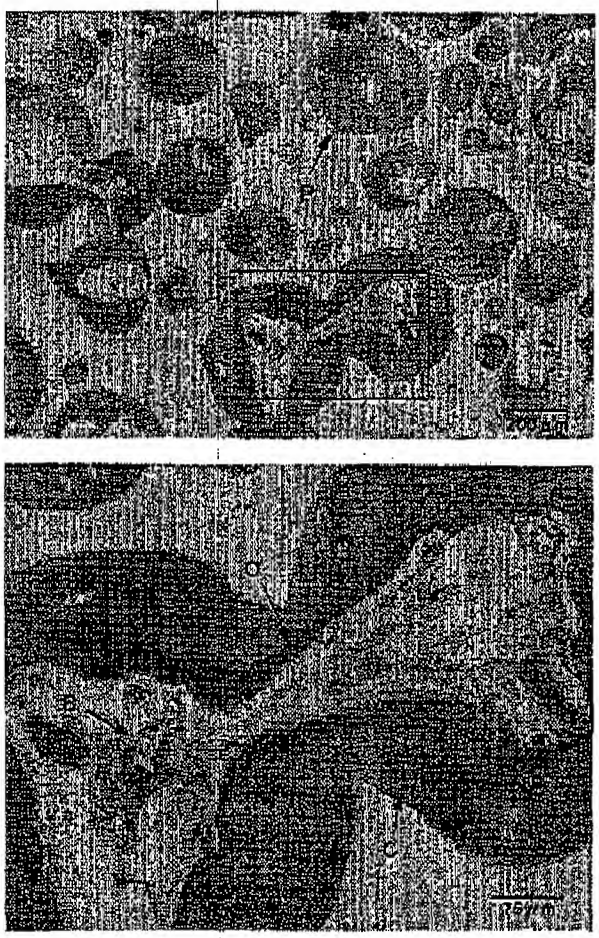
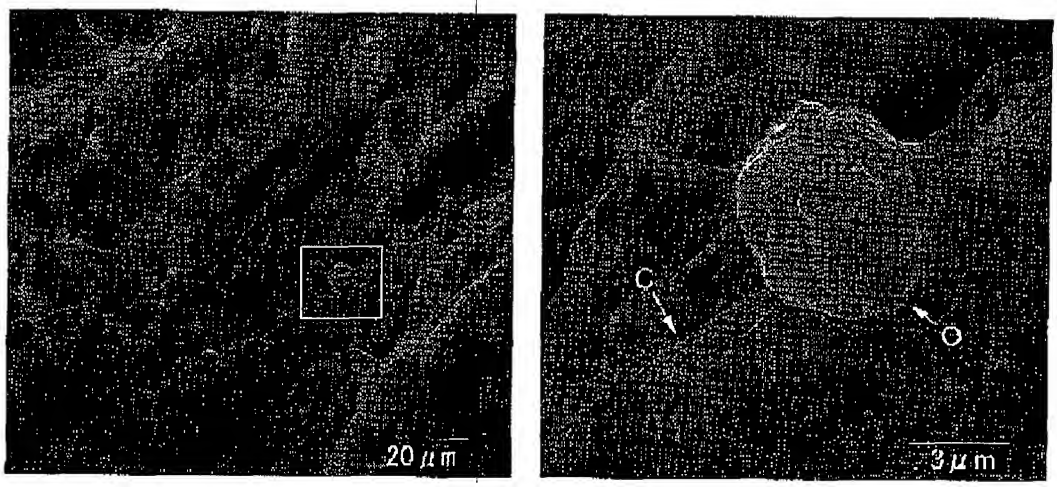


Fig. 3. (a) MSCs-subcultured HA composite 26 weeks after implantation. Hematoxylin and eosin stain. Original magnification ×40 (b) Higher magnification of the large rectangular area in (a). Original magnification ×100.

4. Discussion

The bone marrow stromal system is currently thought to be the reservoir of bone precursor cells. In particular, the mesenchymal component of bone marrow cells can support the self-repair of bone tissue because it contains a low but extremely active fraction of multipotent precursor [15]. After 2 weeks of subculture, the MSCs readily differentiated into osteoblasts and the osteoblasts fabricated mineralized bone matrix on the pore surface of HA [16,17].

In our previous study, we examined six experimental groups pressures ranging from 760 mmHg to 10 mmHg applied to porous HA blocks loaded with MSCs. We found that the level of both ALP activity and OCN content in the 100 mmHg pressure group were highest among the different groups at 4 and 8 weeks after implantation [6]. ALP activity reflects the osteoblastic activity, and bone osteocalcin content



(a) (b)

Fig. 4. SEM photomicrograph of a cross-section of a composite 13 weeks after implantation. (a) The pore surface is covered by osteoblasts as well as collagenous extracellular matrix. Bar = 20 μm. (b) Higher magnification of the large rectangular area in (a). Bar = 3 μm.

is correlated well with the amount of newly formed bone in the composites of HA/MSC after subcutaneous implantation [5]. We considered it necessary for osteoblasts to grow in the presence of a definite oxygen concentration. An appropriate low oxygen concentration can stimulate osteoblast differentiation [18]. However, oxygen concentrations that were too low also injured osteoblasts and decreased the ALP activity.

Appropriate low pressure here had possible effects: removal of air within the pores of the ceramic, and facilitating the flow of cell suspension into the pores. The number of osteogenic cells decides the amount of bone formation to large extent. We speculated that acute hypoxia may have been responsible for some of the effects seen in the present study, although we have no direct evidence for this. Further studies are required to resolve this issue.

Large interconnecting passes between the pores allow MSC to readily enter the central parts of the HA block. The property of a completely interconnected porous biomaterial, which is very similar to cancellous bone matrix, helps fibrovascular tissue invade all pore areas smoothly when grafted *in vivo*. Interconnecting paths of 200 μm in diameter were sufficient for cell and vascular invasion, as also reported by other authors [3]. As shown in Fig. 2, we found many blood vessels in pores, which guarantees further bone formation.

By light microscopy, we found active osteoblasts on the surface of newly formed bone tissue. At different time points, we also found many blood vessels [6]. Scanning electron microscopy revealed many collagenous extracellular matrices with osteoblasts on the surface of pores. These findings indicated that the pressure chosen here was appropriate for osteoblasts to differentiate into osteocytes. No obvious absorption of newly formed bone is observed even after 26 weeks postimplantation.

5. Conclusion

In summary, a novel porous hydroxyapatite, with a cancellous bone-like microstructure, proved to be a good scaffold for osteogenic differentiation of bone marrow-derived mesenchymal stem cells and provided bone-forming biomaterial *in vivo*. Furthermore, applying an appropriate low pressure to HA blocks soaked in a cell suspension benefits increased bone tissue formation even over long periods.

Acknowledgements

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